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Remarks:

The applicant has subsequently filed a sequence
listing and declared, that it includes no new matter.

(54) **Cytochrome bd type quinol oxidase gene of *Brevibacterium lactofermentum***

(57) Oligonucleotides are synthesized based on amino acid sequences of the N-terminus of subunit I, and the N-terminus of subunit II of cytochrome bd type quinol oxidase of *Brevibacterium flavum*. PCR is performed by using the oligonucleotides as primers, and chromosome DNA of *B. flavum* as template, and a gene encoding cytochrome bd type quinol oxidase of *B. flavum* is obtained from a chromosome DNA library of *Brevibacterium lactofermentum* using the above obtained amplification fragment as a probe.

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Description

Technical Field

- 5 [0001] The present invention relates to a cytochrome bd type quinol oxidase of Brevibacterium lactofermentum and a DNA encoding the same.

Background Art

- 10 [0002] Most of organisms acquire energy necessary for life activity by respiration. In higher organisms, carbohydrates, proteins, and aliphatic acids are degraded into acetyl-CoA by the glycolytic pathway and the β -oxidation in cytoplasm, and acetyl-CoA is degraded by the citric acid cycle in mitochondria. The resulting energy is saved as reducing power of NADH and FADH₂. Finally, NADH is completely oxidized to water by the subsequent electron transport system that is present on mitochondrial inner membranes, and a proton concentration gradient is formed in a coupled manner to the
- 15 oxidation, and serves as driving force of the ATP synthesis.

- [0003] Since the bacterial respiratory chain generally comprises various functional enzyme complexes depending on species and growing circumstance, the energy conservation efficiency may vary to a great extent. For example, Escherichia coli contains at least two kinds of quinol oxidases, bo type and bd type, which function as terminal oxidases in the respiratory chain. When a wild-type strain carrying the enzymes of the both types, a mutant strain carrying only
- 20 the bo type, and a mutant strain carrying only the bd type are compared as for growth yield observed in aerobic culture, the growth yield is the lowest in the mutant carrying only the bd type enzyme, and depends on the kind of the terminal oxidases and their energy conservation efficiency (Lecture Abstract for The Conference of The Society for Bioscience and Bioengineering, Japan, 1995, Subject No. 357).

- [0004] Coryneform bacteria such as Brevibacterium lactofermentum and Brevibacterium flavum are gram-positive and aerobic bacteria that are industrially utilized for amino acid producers. Although terminal oxidases of the respiratory chain have been well investigated as for those of Proteobacteria, which is phylogenetically quite far from the coryneform bacteria, and those of Bacillus subtilis and the thermophilic Bacillus, which are also gram-positive bacteria like the coryneform bacteria but phylogenetically somewhat different from them, the electron transport system of respiratory chain in coryneform bacteria has not been investigated in detail. It is considered that it is important to elucidate the elec-
- 30 tron transport system of the respiratory chain, which is the key of the energy metabolism, in coryneform bacteria in view of collecting fundamental data for improving productivity of useful substances. Further, if enzymes involved in the electron transport system of the respiratory chain in coryneform bacteria and genes therefor are identified, they may be useful for, for example, creating strains with higher energy efficiency.

- [0005] To date, it has been reported that the respiration of Brevibacterium lactofermentum is coupled to the proton transport, and it involves cytochromes a, b, and c (Kawahara, Y., et al.(1988) Agric. Biol. Chem. 52(8), 1979-1983). Cytochrome bd type quinol oxidase of Brevibacterium flavum has also been purified and characterized (Kusumoto, Sone and Sakamoto, "Respiratory Chain of Amino Acid Fermenting Bacterium, Brevibacterium flavum, and Characteristics of Its Cytochrome bd Type Menaquinol Oxidase", Abstracts of the 23th Symposium of Bioenergy Study Group, 1997). However, there has not been any report concerning the genes encoding cytochrome bd type quinol oxidase of
- 40 coryneform bacteria.

Description of the Invention

- 45 [0006] The present invention has been accomplished from the aforementioned point of view, and its object is to obtain a gene of cytochrome bd type quinol oxidase of coryneform bacteria, and elucidate its structure.

- [0007] The present inventors synthesized oligonucleotides based on amino acid sequences of the N-terminus of subunit I, and the N-terminus of subunit II of cytochrome bd type quinol oxidase of Brevibacterium flavum, and preformed PCR by utilizing the oligonucleotides as primers, and a chromosomal DNA of Brevibacterium flavum as template to obtain an amplified fragment. Further, they screened a chromosomal DNA library of wild-type Brevibacterium lactofer-
- 50 mentum strain by using the amplified fragment as a probe, and successfully obtained a gene encoding a cytochrome bd type quinol oxidase of Brevibacterium lactofermentum. Thus, the present invention has been completed.

- [0008] That is, the present invention provides:

- (1) a DNA fragment encoding a polypeptide defined in the following (A) or (B):

55

- (A) a polypeptide which has an amino acid sequence shown in SEQ ID NO: 2 of Sequence Listing,
 (B) a polypeptide which has an amino acid sequence shown in SEQ ID NO: 2 of Sequence Listing comprising substitution, deletion, insertion, addition or inversion of one or a plurality of amino acid residues in the amino

acid sequence, and can constitute a protein exhibiting cytochrome bd type quinol oxidase activity together with a subunit II of cytochrome bd type quinol oxidase having an amino acid sequence shown in SEQ ID NO: 4,

(2) a DNA fragment encoding a polypeptide defined in the following (C) or (D):

(C) a polypeptide which has an amino acid sequence shown in SEQ ID NO: 4 of Sequence Listing,

(D) a polypeptide which has an amino acid sequence shown in SEQ ID NO: 4 of Sequence Listing comprising substitution, deletion, insertion, addition or inversion of one or a plurality of amino acid residues in the amino acid sequence, and can constitute a protein exhibiting cytochrome bd type quinol oxidase activity together with a subunit I of cytochrome bd type quinol oxidase having an amino acid sequence shown in SEQ ID NO: 2,

(3) a DNA fragment encoding a polypeptide defined in the following (A) or (B), and a polypeptide defined in the following (C) or (D):

(A) a polypeptide which has an amino acid sequence shown in SEQ ID NO: 2 of Sequence Listing,

(B) a polypeptide which has an amino acid sequence shown in SEQ ID NO: 2 of Sequence Listing comprising substitution, deletion, insertion, addition or inversion of one or a plurality of amino acid residues in the amino acid sequence, and can constitute a protein exhibiting cytochrome bd type quinol oxidase activity together with a subunit II of cytochrome bd type quinol oxidase having an amino acid sequence shown in SEQ ID NO: 4,

(C) a polypeptide which has an amino acid sequence shown in SEQ ID NO: 4 of Sequence Listing,

(D) a polypeptide which has an amino acid sequence shown in SEQ ID NO: 4 of Sequence Listing comprising substitution, deletion, insertion, addition or inversion of one or a plurality of amino acid residues in the amino acid sequence, and can constitute a protein exhibiting cytochrome bd type quinol oxidase activity together with a subunit I of cytochrome bd type quinol oxidase having an amino acid sequence shown in SEQ ID NO: 2,

(4) The DNA of above (1), which is a DNA defined in the following (a) or (b):

(a) a DNA having a nucleotide sequence corresponding to nucleotide numbers 933 to 2483 in the nucleotide sequence depicted in SEQ ID NO: 1 in Sequence Listing; or

(b) a DNA which is hybridizable with the nucleotide sequence of above (a) under a stringent condition, and which codes for a polypeptide which can constitute a protein exhibiting cytochrome bd type quinol oxidase activity together with a subunit II of cytochrome bd type quinol oxidase having an amino acid sequence shown in SEQ ID NO: 4.

(5) The DNA of above (2), which is a DNA defined in the following (c) or (d):

(C) a DNA having a nucleotide sequence corresponding to nucleotide numbers 2476 to 3498 in the nucleotide sequence depicted in SEQ ID NO: 3 in Sequence Listing; or

(d) a DNA which is hybridizable with the nucleotide sequence of above (c) under a stringent condition, and which codes for a polypeptide which can constitute a protein exhibiting cytochrome bd type quinol oxidase activity together with a subunit I of cytochrome bd type quinol oxidase having an amino acid sequence shown in SEQ ID NO: 2.

(6) The DNA of above (3), which comprising a DNA defined in the following (a) or (b), and a DNA defined in the following (c) or (d):

(a) a DNA having a nucleotide sequence corresponding to nucleotide numbers 933 to 2483 in the nucleotide sequence depicted in SEQ ID NO: 1 in Sequence Listing; or

(b) a DNA which is hybridizable with the nucleotide sequence of above (a) under a stringent condition, and which codes for a polypeptide which can constitute a protein exhibiting cytochrome bd type quinol oxidase activity together with a subunit II of cytochrome bd type quinol oxidase having an amino acid sequence shown in SEQ ID NO: 4; and

(c) a DNA having a nucleotide sequence corresponding to nucleotide numbers 2476 to 3498 in the nucleotide sequence depicted in SEQ ID NO: 3 in Sequence Listing; or

(d) a DNA which is hybridizable with the nucleotide sequence of above (c) under a stringent condition, and which codes for a polypeptide which can constitute a protein exhibiting cytochrome bd type quinol oxidase activity together with a subunit I of cytochrome bd type quinol oxidase having an amino acid sequence shown in SEQ ID NO: 2.

(7) a DNA fragment defined in the above (1) which has a nucleotide sequence comprising nucleotides of the nucleotide numbers 933 to 2483 in the nucleotide sequence shown in SEQ ID NO: 1,

(8) a DNA fragment defined in the above (2) which has a nucleotide sequence comprising nucleotides of the nucleotide numbers 2476 to 3498 in the nucleotide sequence shown in SEQ ID NO: 1, and

(9) a DNA fragment defined in the above (3) which has a nucleotide sequence comprising nucleotides of the nucleotide numbers 933 to 3498 in the nucleotide sequence shown in SEQ ID NO: 1.

[0009] In the present description, the term cytochrome bd type quinol oxidase activity means activity exhibiting oxidoreduction differential absorption spectra of cytochrome b and cytochrome d, which is for oxidizing a reduced type quinone compounds (quinols) with consumption of oxygen. A DNA fragment that encodes cytochrome bd type quinol oxidase or a subunit thereof will be referred to as the "DNA of the present invention" as the case may be.

Brief Description of the Drawings

[0010]

Figure 1 represents the results of hydropathy analysis of subunits I of cytochrome bd type quinol oxidases of *Brevibacterium lactofermentum*, *Bacillus stearothermophilus* and *Escherichia coli*. The symbol "*" indicates an amino acid residue shared by the three oxidases.

Figure 2 represents amino acid sequence alignment of subunits I of cytochrome bd type quinol oxidases of *Brevibacterium lactofermentum*, *Bacillus stearothermophilus* and *Escherichia coli*.

Figure 3 represents amino acid sequence alignment of subunits II of cytochrome bd type quinol oxidases of *Brevibacterium lactofermentum*, *Bacillus stearothermophilus* and *Escherichia coli*.

Detailed Description of the Invention

[0011] The present invention will be explained in more detail hereinafter.

[0012] The DNA of the present invention can be obtained from *B. lactofermentum* chromosomal DNA based on partial amino acid sequences of cytochrome bd type quinol oxidase of *B. flavum*. Specifically, PCR is performed by using oligonucleotides synthesized based on the amino acid sequences as primers, and chromosomal DNA of *B. flavum* as template to obtain a partial sequence of cytochrome bd type quinol oxidase gene of *B. flavum*. Then, by screening a chromosomal DNA library of *B. lactofermentum* using the obtained partial sequence as a probe, a gene encoding cytochrome bd type quinol oxidase of *B. lactofermentum* can be obtained.

[0013] Chromosomal DNA of *B. flavum* and *B. lactofermentum* can be prepared by, for example, the method of Saito and Miura (*Biochem. Biophys. Acta.*, 72, 619, (1963)), and the method of K. S. Kirby (*Biochem. J.*, 64, 405, (1956)). A chromosome DNA library can be obtained by partially digesting chromosomal DNA with a suitable restriction enzyme, ligating each of the obtained DNA fragments to a vector DNA autonomously replicable in *Escherichia coli* cell to prepare a recombinant DNA, and introducing the DNA into *E. coli*. The vector is not particularly limited, so long as it is a vector usually used for genetic cloning, and plasmid vectors such as pUC19, pUC18, pUC118, and pUC119, phage vectors such as lambda phage DNA and the like can be used.

[0014] The primer used for the PCR may be, for example, an oligonucleotide having a nucleotide sequence shown in SEQ ID NO: 7 or SEQ ID NO: 8. In order to confirm that an obtained PCR product has a desired sequence, it can be confirmed that it contains a sequence corresponding to the primer by nucleotide sequencing, or confirming that the amino acid sequence deduced from the nucleotide sequence contains a partial amino acid sequence of cytochrome bd type quinol oxidase of *B. flavum*.

[0015] The screening of a chromosome DNA library of *B. lactofermentum* utilizing the DNA fragment obtained in the PCR as a probe can be performed by colony hybridization when plasmid vectors are used for the preparation of the library, or plaque hybridization when phage vectors are used for the preparation of the library. A hybridization positive clone can be confirmed to contain a purpose cytochrome bd type quinol oxidase gene by nucleotide sequencing of DNA prepared from the clone. It is also possible to preliminarily perform Southern analysis for a hybridization positive clone by using the probe.

[0016] A nucleotide sequence of cytochrome bd type quinol oxidase gene of *B. lactofermentum* ATCC 13869 strain obtained in the working example in such a manner as described above is shown in SEQ ID NO: 1. Expected coding regions and amino acid sequences of proteins encoded thereby are shown in SEQ ID NOS: 1-4. Estimation of coding regions and operon structure and analysis of homology to cytochrome bd type quinol oxidases of *Bacillus stearothermophilus* K1041 and *Escherichia coli* were performed by using GENETYX. Homology Version 2.2.2 (Software Devel-

opment Co., Ltd.).

[0017] The cytochrome bd type quinol oxidase gene contains two open reading frames (cydA and cydB reading from the 5' end), and they encode subunit I or cytochrome bd type quinol oxidase (also referred to as merely "subunit I" hereinafter) and subunit II of the same (also referred to as merely "subunit II" hereinafter), respectively. It was estimated that cydA and cydB comprised 1551 bp and 1023 bp respectively, the subunit I consisted of 517 amino acid residues, and the subunit II consisted of 341 amino acid residues. A promoter-like sequence was present upstream of cydA, an SD-like sequence was present upstream of each of cydA and cydB, and a terminator-like sequence was present downstream of cydB. Therefore, it was considered that cydA and cydB formed a cyd operon.

[0018] While the codon of the N-terminal amino acid residue of the subunit I is indicated as GTG, and the corresponding amino acid as Val in Sequence Listing, it is actually Met. This is considered to be caused because GTG is recognized as an initiation methionine. Such cases have been reported elsewhere.

[0019] Figures 1 and 2 represent the results of hydropathy analysis performed for comparison of structures of the cytochrome bd type quinol oxidase of the present invention and subunits I of Bacillus stearothermophilus and E. coli, and alignment of the amino acid sequences. The indications I-VII represent transmembrane helix regions, and therefore it was confirmed that there were at least seven transmembrane helices. It can be understood from the patterns shown in the graphs that they resemble each other. Further, a region containing a quinol binding site called Q loop was present between V and VI of the subunit I of E. coli, whereas there was no region exhibiting homology with the latter half portion of the Q loop in B. lactofermentum like B. stearothermophilus cydA, and hence the Q loop region was shortened. Considering this point, it is expected that cytochrome bd type quinol oxidase of B. lactofermentum has a structure more similar to that of cytochrome bd type quinol oxidase of B. stearothermophilus rather than that of E. coli. The comparison of amino acid sequences of the subunit I showed that B. lactofermentum had about 24.7% homology to B. stearothermophilus and, about 38.6% to E. coli, and it was considered that, as for the subunit I as a whole, cytochrome bd type quinol oxidase of B. lactofermentum has a structure more similar to cytochrome bd type quinol oxidase of E. coli rather than that of B. stearothermophilus.

[0020] There have been reported H19, H186, and M393 for E. coli cydA, and H21, H184, and M326 for B. stearothermophilus cydA as functionally important residues in view of being a ligand of hem b558. These amino acids are conserved also in cydA of B. lactofermentum as H18, H185, and M350.

[0021] Figure 3 represents alignment of amino acid sequences of the three kinds of bacterial subunits II. As for the subunit II, B. lactofermentum showed about 25.9% homology to B. stearothermophilus, and about 34.8% to E. coli.

[0022] The DNA of the present invention is a DNA encoding the subunit I, which is encoded by the nucleotide sequence shown in SEQ ID NO: 2, the subunit II, which is encoded by the nucleotide sequence shown in SEQ ID NO: 4, or cytochrome bd type quinol oxidase protein containing these subunit I and subunit II. The subunit I, subunit II or cytochrome bd type quinol oxidase protein can be produced by introducing such a DNA into a suitable host cell, and culturing the obtained transformant so that the DNA should be expressed. A DNA having a nucleotide sequence comprising nucleotides of the nucleotide numbers 933-2483 in the nucleotide sequence shown in SEQ ID NO: 1 can be mentioned as a DNA encoding the subunit I, a DNA having a nucleotide sequence comprising nucleotides of the nucleotide numbers 2476-3498 as a DNA encoding the subunit II, and a DNA having a nucleotide sequence comprising nucleotides of the nucleotide numbers 933-3498 as a DNA encoding the both.

[0023] The produced cytochrome bd type quinol oxidase protein or a subunit thereof can be collected and purified from culture by a method commonly used for the purification of proteins such as salting out, solvent precipitation, gel filtration chromatography, and ion exchange chromatography.

[0024] The DNA of the present invention encoding the subunit I may be either one encoding a polypeptide having an amino acid sequence shown in SEQ ID NO: 2 comprising substitution, deletion, insertion, addition or inversion of one or a plurality of amino acid residues in the amino acid sequence, or a polypeptide that can constitute a protein exhibiting cytochrome bd type quinol oxidase activity together with the subunit II

[0025] The DNA of the present invention encoding the subunit II may be either one encoding a polypeptide having an amino acid sequence shown in SEQ ID NO: 4 comprising substitution, deletion, insertion, addition or inversion of one or a plurality of amino acid residues in the amino acid sequence, or a polypeptide that can constitute a protein exhibiting cytochrome bd type quinol oxidase activity together with the subunit I.

[0026] Further, a DNA encoding a cytochrome bd type quinol oxidase which contains mutations in the subunit I, the subunit II or the both is also included in the DNA of the present invention.

[0027] The term "a plurality of amino acid residues" preferably means 1-40, more preferably 1-10 amino acid residues.

[0028] DNA, which codes for the substantially same protein as subunit I and/of the subunit II as described above, is obtained, for example, by modifying the nucleotide sequence, for example, by means of the site-directed mutagenesis method so that one or more amino acid residues at a specified site involve substitution, deletion, insertion, addition, or inversion. DNA modified as described above may be obtained by the conventionally known mutation treatment. The mutation treatment includes a method for treating DNA coding for subunit I and/of the subunit II in vitro, for example, with hydroxylamine, and a method for treating a microorganism, for example, a bacterium belonging to the genus

Escherichia harboring DNA coding for subunit I and/of the subunit II with ultraviolet irradiation or a mutating agent such as N-methyl-N'-nitro-N-nitrosoguanidine (NTG) and nitrous acid usually used for the mutation treatment.

[0029] The substitution, deletion, insertion, addition, or inversion or nucleotide as described above also includes mutation (mutant or variant) which naturally occurs, for example, on the basis of the individual difference or the difference in species or genus or coryneform bacteria which harbors cytochrome bd type quinol oxidase.

[0030] The DNA, which codes for substantially the same protein as subunit I and/of the subunit II, is obtained by expressing DNA having mutation as described above in an appropriate cell, and investigating an activity of an expressed product. The DNA, which codes for substantially the same protein as subunit I and/of the subunit II, is also obtained by isolating DNA which is hybridizable with DNA having, for example, a nucleotide sequence corresponding to nucleotide numbers of 933 to 2483 of the nucleotide sequence depicted in SEQ ID NO: 1 and/or a nucleotide sequence corresponding to nucleotide numbers of 2476 to 3498 of the nucleotide sequence depicted in SEQ ID NO: 3 in Sequence Listing under a stringent condition, and which codes for a protein having the activity of subunit I and/or subunit II, from DNA coding for subunit I and/or subunit II having mutation or from a cell harboring it.

[0031] The "stringent condition" referred to herein is a condition under which so-called specific hybrid is formed, and non-specific hybrid is not formed. It is difficult to clearly express this condition by using any numerical value. However, for example, the stringent condition includes a condition under which DNA's having high homology, for example, DNA's having homology of not less than 50 % are hybridized with each other, and DNA's having homology lower than the above are not hybridized with each other. Alternatively, the stringent condition is exemplified by a condition under which DNA's are hybridized with each other at a salt concentration corresponding to an ordinary condition of washing in Southern hybridization, i.e., 60°C, 1 x SSC, 0.1 % SDS, preferably 0.1 x SSC, 0.1 % SDS.

[0032] The gene, which is hybridizable under the condition as described above, includes those having a stop codon generated within a coding region of the gene, and those having no activity due to mutation of active center. However, such inconveniences can be easily removed by ligating the gene with a commercially available activity expression vector, and investigating cytochrome bd type quinol oxidase activity.

[0033] The host for the expression of the DNA of the present invention include, for example, various kinds of bacteria including *E. coli*, coryneform bacteria such as *B. lactofermentum* and *B. flavum*, eucaryotic cells such as *Saccharomyces cerevisiae* and the like. In order to introduce the DNA of the present invention into a host such as those mentioned above, the host cell can be transformed with a recombinant vector which is obtained by inserting the DNA of the present invention into a vector selected depending on the kind of the host in which the expression is to be obtained. Those procedures can be performed by using methods of genetic recombination well known to those skilled in the art.

[0034] The DNA of the present invention and cytochrome bd type quinol oxidase or the subunits thereof encoded thereby are considered to be useful for elucidating the electron transport system of coryneform bacteria. The DNA of the present invention is also expected to be utilized for breeding of coryneform bacteria producing useful substances with high energy efficiency.

Best Mode for Carrying out the Invention

[0035] The present invention will be specifically explained with reference to the following examples.

(1) Purification of cytochrome bd type quinol oxidase of *Brevibacterium flavum*

[0036] Bacterial cells (about 120 g wet weight) of *B. flavum* ATCC 14067 strain that had been cultivated by the end of the stationary phase were suspended in 200 ml of a buffer (0.5% NaCl, 10 mM sodium phosphate, pH 7.4), and immediately disrupted by stirring at a high speed by means of a bead beater (Biospec) in the presence of 0.5 mM of glass particles. After this suspension of disrupted cells was centrifuged at 5,000 rpm for 10 minutes to remove undisrupted bacterial cells, the supernatant was subjected to centrifugation at 15,000 rpm for 10 minutes and the resulting supernatant was further subjected to centrifugation at 15,000 rpm for 30 minutes. The precipitates obtained in the both centrifugations were combined, and suspended in the same buffer as mentioned above to obtain a membrane preparation.

[0037] The above membrane preparation (5 mg/ml, 0.5% NaCl, 10 mM sodium phosphate, pH 7.4) was homogenized by a Teflon homogenizer, and centrifuged at 40,000 rpm for 20 minutes, and precipitates were collected. The precipitates were added with 1.5% sodium cholate, 0.5% sodium deoxycholate, 0.1% NaCl, and 10 mM sodium phosphate (pH 7.4), then homogenized and centrifuged at 40,000 rpm for 20 minutes to collect the precipitates. The precipitates were further added with 10 mM sodium phosphate (pH 7.4), homogenized, and centrifuged at 40,000 rpm for 20 minutes to collect the precipitates.

[0038] The membrane preparation washed with cholic acid as described above was suspended in a buffer containing surface active agents, n-nonanoyl-N-methylglucamide (MEGA-9) and decanoyl-N-methylglucamide (MEGA-10) each at 1%. This suspension was homogenized on ice, sonicated, and centrifuged at 40,000 rpm for 20 minutes to obtain a supernatant.

[0039] The above supernatant obtained by the centrifugation was adsorbed on a hydroxyapatite column equilibrated with 1% MEGA-9, 1% MEGA-10, 10% glycerol, and 10 mM sodium phosphate (pH 7.4), and fractionated by elution with a concentration of sodium phosphate increased stepwise (0, 50, 150, 250, and 400 mM). Cytochromes in the fractions were detected by reduced minus oxidized difference spectrum. As a result, cytochromes c and b were detected in the fraction eluted at 50 mM of sodium phosphate, cytochromes c, b and a in the fraction eluted at 150 mM, and cytochromes b and d in the fraction eluted at 250 mM.

[0040] The fraction eluted at a sodium phosphate concentration of 250 mM was dialyzed against 10% glycerol and 10 mM sodium phosphate (pH 7.4), then adsorbed on a DEAE-Toyopearl (Tohso) column equilibrated with the same buffer, and fractionated by elution with a concentration of NaCl increased stepwise (0, 80, 100, 120, 140, and 300 mM). Cytochromes in the fractions were detected by reduced minus oxidized difference spectrum. As a result, cytochromes b and d were detected in the fraction eluted at a NaCl concentration of 120 mM. This fraction was used as cytochrome bd type quinol oxidase enzyme preparation.

[0041] The above enzyme preparation was subjected to SDS-polyacrylamide gel electrophoresis using 13.5% gel, and blotted on a PVDF membrane. Portions of the membrane corresponding to the subunit I and the subunit II were subjected to amino acid sequence analysis to determine the N-terminal amino acid sequences. The amino acid sequences are shown in SEQ ID NO: 5 (subunit I) and SEQ ID NO: 6 (subunit II), respectively.

(2) Isolation of cytochrome bd type quinol oxidase gene of *Brevibacterium lactofermentum*

[0042] Screening of a chromosome DNA library of *B. lactofermentum* for clones containing cytochrome bd type quinol oxidase gene was performed by colony hybridization.

[0043] Two kinds of oligonucleotides were synthesized based on the above partial amino acid sequences of cytochrome bd type quinol oxidase of *B. flavum*. One was prepared based on the N-terminal amino acid sequence of the subunit I of cytochrome bd type quinol oxidase (bbd1: SEQ ID NO: 7), and the other was prepared based on the N-terminal amino acid sequence of the subunit II (bbd2: SEQ ID NO: 8).

[0044] PCR was performed by using the above primers bbd1 and bbd2 and chromosome DNA of the strain ATCC 14067 as template. As for the reaction condition, after denaturation at 94°C for one minute, a cycle comprising denaturation at 95°C for 45 seconds, annealing at 50°C for 60 seconds, and chain extension reaction at 62°C for 90 seconds was repeated for 35 cycles. As a result, fragments of about 1500 bp, 800 bp, and 100 bp were provided. Based on the molecular weight 56.4 kD of the subunit I estimated from the purified protein, and the reported molecular weights of subunits I of cytochrome bd type quinol oxidases of other bacteria, the fragment of about 1500 bp was considered to be the desired PCR product. Therefore, the PCR product was electrophoresed on 2% agarose gel, and a portion of about 1.5 kbp fragment was excised from the gel to extract the DNA.

[0045] This DNA fragment was blunt-ended by using DNA Blunting Kit (Takara Shuzo), and ligated to pUC118 vector digested with *Sma*I and treated with alkaline phosphatase by using DNA ligation Kit Ver. 2 (Takara Shuzo). *E. coli* XL-1 Blue strain was transformed with the obtained recombinant primer.

[0046] Plasmid was prepared from the obtained transformant, and the inserted nucleotide sequence was determined. The nucleotide sequencing was performed by using Fluorescein Labeled Primer M4 (Takara Shuzo, SEQ ID NO: 9) as the forward primer, and Fluorescein Labeled Primer RV-MF (Takara Shuzo, SEQ ID NO: 10) as the reverse primer according to the protocol of Thermo Sequence fluorescent labelled primer cycle sequencing kit (Amersham Life Science). As a result, it was confirmed that the cytochrome bd type quinol oxidase gene was contained in the plasmid based on the homology with the primer. This partial clone was designated BD1.

[0047] This BD1 was amplified by PCR using the aforementioned primers M4 and RV-M, and a probe labeled with DIG (digoxigenin) was prepared by using DIG DNA Labeling Kit (Boehringer Mannheim).

[0048] Chromosomal DNA library of *B. lactofermentum* was screened by using the aforementioned probe. The library was obtained by partially digesting chromosomal DNA of *B. lactofermentum* ATCC 13869 with *Sau*3A1, inserting the product into *Bam*HI site of pUC18, and transforming *E. coli* XL-1 Blue with the obtained recombinant plasmid. Colony hybridization was performed for the colonies of transformants by using the probe labeled with DIG mentioned above. The detection of the probe was performed by using DIG Detection Kit (Boehringer Mannheim) which utilized anti-DIG antibodies labeled with alkaline phosphatase.

[0049] Plasmid was prepared from hybridization positive colonies, digested with *Eco*RI and *Pst*I, and subjected to Southern blotting using BD1 as a probe. As a result, two positive clones were obtained. Inserted fragments of these positive clones were designated BD21 and BD31, respectively. BD21 comprised about 3.8 kbp, and BD31 comprised about 9.0 kbp. These clones were subcloned, and their nucleotide sequences were determined. The results are shown in SEQ ID NO: 1. Expected coding regions and amino acid sequences of the proteins encoded thereby are shown in SEQ ID NOS: 1-4.

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SEQUENCE LISTING

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10 Brevibacterium lactofermentum

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	Gly Asp Val Phe Gly Gly Pro Leu Ala Leu Glu Gly Leu Ile Ala Phe				
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	Phe Leu Glu Ser Val Phe Leu Gly Leu Trp Ile Phe Gly Trp Gly Lys				
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	Ile Pro Gly Trp Leu His Thr Ala Ser Ile Trp Ile Val Ala Ile Ala				
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	Thr Asn Ile Ser Ala Tyr Phe Ile Ile Val Ala Asn Ser Phe Met Gln				
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	His Pro Val Gly Ala Glu Tyr Asn Pro Glu Thr Gly Arg Ala Glu Leu				
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	Thr Asp Phe Trp Ala Leu Leu Thr Asn Ser Thr Ala Leu Ala Ala Phe				
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EP 0 967 282 A2

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EP 0 967 282 A2

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20	Trp Ile Phe Gly Trp Gly Lys Ile Pro Gly Trp Leu His Thr Ala Ser		
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25	Ile Trp Ile Val Ala Ile Ala Thr Asn Ile Ser Ala Tyr Phe Ile Ile		
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30	Val Ala Asn Ser Phe Met Gln His Pro Val Gly Ala Glu Tyr Asn Pro		
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35	Glu Thr Gly Arg Ala Glu Leu Thr Asp Phe Trp Ala Leu Leu Thr Asn		
	165	170	175
40	Ser Thr Ala Leu Ala Ala Phe Pro His Ala Val Ala Gly Gly Phe Leu		
	180	185	190
45	Thr Ala Gly Thr Phe Val Leu Gly Ile Ser Gly Trp Trp Ile Ile Arg		
	195	200	205
50	Ala His Arg Gln Ala Lys Lys Ala Glu Ala Glu Ile Glu Ser Lys His		
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55	Ser Met His Arg Pro Ala Leu Trp Val Gly Trp Trp Thr Thr Val Val		
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60	Ser Ser Val Ala Leu Phe Ile Thr Gly Asp Thr Gln Ala Lys Leu Met		
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Thr Ala Thr Asp Pro Asn Phe Ser Ile Leu Thr Ile Gly Thr His Asn
275 280 285

Asn Cys Asp Thr Val Thr His Leu Ile Asp Val Pro Phe Val Leu Pro
290 295 300

Phe Leu Ala Glu Gly Lys Phe Thr Gly Val Thr Leu Gln Gly Val Asn
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Gln Leu Gln Ala Ala Ala Glu Gln Ala Tyr Gly Pro Gly Asn Tyr Ser
325 330 335

Pro Asn Leu Phe Val Thr Tyr Trp Ser Phe Arg Ala Met Ile Gly Leu
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Met Leu Gly Ser Leu Ala Ile Ala Ala Ile Ala Trp Leu Leu Arg
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Lys Lys Arg Thr Pro Thr Gly Lys Ile Ala Arg Leu Phe Gln Ile Gly
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Ser Leu Ile Ala Ile Pro Phe Pro Phe Leu Ala Asn Ser Ala Gly Trp
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Met Gly Val Ser Asp His Ala Pro Trp Gln Val Trp Leu Thr Leu Ile
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EP 0 967 282 A2

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EP 0 967 282 A2

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Thr Met Phe Ser Gly Met Tyr Leu Pro Leu Phe Leu Val Leu Val Ser
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EP 0 967 282 A2

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230

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295

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3423

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EP 0 967 282 A2

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Cys Leu Lys Val Gly Lys Ile Glu Tyr
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EP 0 967 282 A2

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 30 Met Trp Gly Phe Ile Phe Ala Asn Ile Phe Lys Leu Ala Cys Pro Ser
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 35 Arg Arg Ile Thr Pro Ser Met Leu Gln Trp Leu Cys Cys Ala Met Phe
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 40 Asn Val Phe Ala Ile Leu Gly Ala Leu Ala Phe Thr Ala Leu Phe Ala
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 45 Leu His Gly Leu Ala Phe Ile Arg Leu Lys Thr Ala Gly Arg Val Arg
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 50 Thr Asp Ala Ala Lys Ala Ala Pro Val Val Ala Leu Leu Ala Ala Val
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 55 Thr Gly Gly Pro Phe Val Leu Trp Ala Ala Ile Ala Tyr Gly Arg Ser
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Trp Ser Trp Ile Leu Ala Val Leu Ile Ile Ala Ala Val Leu Gly Gly
225 230 235 240

Ala Phe Ala Leu Ile Lys Asp Arg Asp Gly Leu Ser Phe Leu Ser Thr
245 250 255

Ser Val Ala Val Ile Gly Val Val Ala Leu Leu Phe Ser Ser Leu Phe
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Pro Asn Val Met Pro Thr Thr Leu Ala Asp Gly Val Thr Gly Tyr Leu
275 280 285

Glu Arg Leu Arg Lys Pro Leu Arg Ile Asp His Pro Asp Leu Asp Arg
290 295 300

His Cys Asp Arg Thr Ala Gly Cys Pro Leu Pro Arg Leu Asp Leu Leu
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Gly Lys Ile Glu Tyr
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5 ttgtgaccta tcacaaggct gatgatgtat ccgaagtact cgttaccagg atgaactcgt 360

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ggccttgatt tcttctacct tggtaacggt cattcagaaa acagcaaaca gtcacgatg 540

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agcaaaatca gtcattgaca gggaaaggtt gaccacaatc ggggtagcc tttctaaagt 780

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tcaatctgta aagaagagga aggggacctc gc gtg gat gtc gtt gac atc gcg 953

Val Asp Val Val Asp Ile Ala

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cgg tgg caa ttc gga att acc acc gtc tat cac ttc att ttt gtc cca 1001

40 Arg Trp Gln Phe Gly Ile Thr Thr Val Tyr His Phe Ile Phe Val Pro

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ctg acc att ggc tta gca ccg ctg gtc gcg atc atg caa acg ttt tgg 1049

45 Leu Thr Ile Gly Leu Ala Pro Leu Val Ala Ile Met Gln Thr Phe Trp

25 30 35

caa gtt acc ggc aaa gag cac tgg tat cgg gct acg aga ttt ttt ggc 1097

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40 45 50 55

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	act gtg ctg ctc atc aac ttc gcg gtt ggt gta gca acg ggc att gtg	1145
	Thr Val Leu Leu Ile Asn Phe Ala Val Gly Val Ala Thr Gly Ile Val	
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	cag gag ttc cag ttc ggt atg aac tgg tcg gaa tat tcg cgt ttc gtc	1193
	Gln Glu Phe Gln Phe Gly Met Asn Trp Ser Glu Tyr Ser Arg Phe Val	
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	ggg gat gtt ttc ggc gga ccg ctg gct ttg gag ggg ctc atc gcg ttc	1241
	Gly Asp Val Phe Gly Gly Pro Leu Ala Leu Glu Gly Leu Ile Ala Phe	
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	ttc ctt gag tct gtg ttc tta ggt ctg tgg att ttc gga tgg ggg aag	1289
	Phe Leu Glu Ser Val Phe Leu Gly Leu Trp Ile Phe Gly Trp Gly Lys	
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	att cct gga tgg ctg cat act gcg tcc att tgg atc gtt gct att gcg	1337
	Ile Pro Gly Trp Leu His Thr Ala Ser Ile Trp Ile Val Ala Ile Ala	
25	120 125 130 135	
	acg aat att tct gcc tat ttc atc atc gtg gcc aac tcg ttt atg cag	1385
	Thr Asn Ile Ser Ala Tyr Phe Ile Ile Val Ala Asn Ser Phe Met Gln	
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	cat ccg gtg ggt gct gag tat aac cct gag act ggt cgg gcg gag ctt	1433
	His Pro Val Gly Ala Glu Tyr Asn Pro Glu Thr Gly Arg Ala Glu Leu	
35	155 160 165	
	act gat ttc tgg gct ctt ctc aca aac tcc acc gcg ctg gct gcg ttc	1481
	Thr Asp Phe Trp Ala Leu Leu Thr Asn Ser Thr Ala Leu Ala Ala Phe	
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	ccg cat gct gtt gcc ggt ggt ttt tta aca gct gga act ttc gtc ctc	1529
	Pro His Ala Val Ala Gly Gly Phe Leu Thr Ala Gly Thr Phe Val Leu	
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	Gly Ile Ser Gly Trp Trp Ile Ile Arg Ala His Arg Gln Ala Lys Lys	
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	gct gag gcg gaa atc gag tcg aag cat tca atg cac agg ccg gcg ttg	1625
	Ala Glu Ala Glu Ile Glu Ser Lys His Ser Met His Arg Pro Ala Leu	
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	tgg gtt ggt tgg tgg acc aca gtt gtc tct tcc gtg gca ctg ttc atc	1673
	Trp Val Gly Trp Trp Thr Thr Val Val Ser Ser Val Ala Leu Phe Ile	
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	act ggc gat aca cag gcg aag ctc atg ttc gtg cag cag ccg atg aag	1721
	Thr Gly Asp Thr Gln Ala Lys Leu Met Phe Val Gln Gln Pro Met Lys	
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	atg gcg tcg gcg gaa tcc ttg tgt gaa acc gcc aca gat cca aac ttc	1769
	Met Ala Ser Ala Glu Ser Leu Cys Glu Thr Ala Thr Asp Pro Asn Phe	
25	265 270 275	
	tcc att ctg aca att ggt acg cac aac aac tgc gat acg gta acc cac	1817
	Ser Ile Leu Thr Ile Gly Thr His Asn Asn Cys Asp Thr Val Thr His	
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	ctg atc gat gtt ccg ttt gtg ctt cca ttc ttg gct gaa gga aaa ttc	1865
	Leu Ile Asp Val Pro Phe Val Leu Pro Phe Leu Ala Glu Gly Lys Phe	
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	acc ggt gtg act ttg cag ggt gta aac cag ctc caa gct gca gcg gag	1913
	Thr Gly Val Thr Leu Gln Gly Val Asn Gln Leu Gln Ala Ala Glu	
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	caa gca tac ggt cct ggc aac tac tcc cct aac ttg ttt gtc acc tac	1961
	Gln Ala Tyr Gly Pro Gly Asn Tyr Ser Pro Asn Leu Phe Val Thr Tyr	
45	330 335 340	
	tgg tca ttc cgc gca atg atc ggc cta atg ctt ggt tct ttg gct atc	2009
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Trp Ser Phe Arg Ala Met Ile Gly Leu Met Leu Gly Ser Leu Ala Ile
345 350 355

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gct gcg att gcg tgg ctg ttg ctg cgt aag aag cgc aca cca act gga 2057
Ala Ala Ile Ala Trp Leu Leu Leu Arg Lys Lys Arg Thr Pro Thr Gly
360 365 370 375

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aag att gct cgt ctc ttc caa atc ggc agc ctc att gcc att cca ttc 2105
Lys Ile Ala Arg Leu Phe Gln Ile Gly Ser Leu Ile Ala Ile Pro Phe
380 385 390

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cca ttc ttg gct aac tct gct ggt tgg atc ttc acc gag atg ggc cgc 2153
Pro Phe Leu Ala Asn Ser Ala Gly Trp Ile Phe Thr Glu Met Gly Arg
395 400 405

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cag cct tgg gtg gta cac ccg aat cct gaa tct gcc ggc gat gcc cga 2201
Gln Pro Trp Val Val His Pro Asn Pro Glu Ser Ala Gly Asp Ala Arg
410 415 420

25

aca gag atg atc cgg atg act gtt gat atg ggt gtg tct gat cat gcg 2249
Thr Glu Met Ile Arg Met Thr Val Asp Met Gly Val Ser Asp His Ala
425 430 435

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ccg tgg caa gtc tgg ctg act cta att ggc ttc acg att ctc tat ctc 2297
Pro Trp Gln Val Trp Leu Thr Leu Ile Gly Phe Thr Ile Leu Tyr Leu
440 445 450 455

35

atc ttg ttc gtg gtg tgg gtg tgg ctg att cgc cgc gca gtt ctg atc 2345
Ile Leu Phe Val Val Trp Val Trp Leu Ile Arg Arg Ala Val Leu Ile
460 465 470

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gga cca cca gag gaa ggc gct cca tcc gtg gag gca aag act gga ccg 2393
Gly Pro Pro Glu Glu Gly Ala Pro Ser Val Glu Ala Lys Thr Gly Pro
475 480 485

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gca acc ccg att ggt tca gat atg ccc atg aca ccg ctg caa ttt acc 2441
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	505	510	515	
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	attcgacttc ggtgtcggaa ttttagcgcc gatcatcggg aaagattccg ccgctaaaaa			2603
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45 Ala Ile Met Gln Thr Phe Trp Gln Val Thr Gly Lys Glu His Trp Tyr
35 40 45
50 Arg Ala Thr Arg Phe Phe Gly Thr Val Leu Leu Ile Asn Phe Ala Val
50 55 60
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5 Gly Val Ala Thr Gly Ile Val Gln Glu Phe Gln Phe Gly Met Asn Trp
65 70 75 80

10 Ser Glu Tyr Ser Arg Phe Val Gly Asp Val Phe Gly Gly Pro Leu Ala
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15 Leu Glu Gly Leu Ile Ala Phe Phe Leu Glu Ser Val Phe Leu Gly Leu
100 105 110

20 Trp Ile Phe Gly Trp Gly Lys Ile Pro Gly Trp Leu His Thr Ala Ser
115 120 125

25 Ile Trp Ile Val Ala Ile Ala Thr Asn Ile Ser Ala Tyr Phe Ile Ile
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30 Val Ala Asn Ser Phe Met Gln His Pro Val Gly Ala Glu Tyr Asn Pro
145 150 155 160

35 Glu Thr Gly Arg Ala Glu Leu Thr Asp Phe Trp Ala Leu Leu Thr Asn
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40 Ser Thr Ala Leu Ala Ala Phe Pro His Ala Val Ala Gly Gly Phe Leu
180 185 190

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50 Ala His Arg Gln Ala Lys Lys Ala Glu Ala Glu Ile Glu Ser Lys His
210 215 220

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225 230 235 240

Ser Ser Val Ala Leu Phe Ile Thr Gly Asp Thr Gln Ala Lys Leu Met
245 250 255

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 Phe Leu Ala Glu Gly Lys Phe Thr Gly Val Thr Leu Gln Gly Val Asn
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 Pro Asn Leu Phe Val Thr Tyr Trp Ser Phe Arg Ala Met Ile Gly Leu
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 Lys Lys Arg Thr Pro Thr Gly Lys Ile Ala Arg Leu Phe Gln Ile Gly
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	Gly Ile Leu Ala Pro Ile Ile Gly Lys Asp Ser Ala Ala Lys Asn Thr	
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	atc atc cgc acc atc ggc cct gtc tgg gac gga aat gaa gtg tgg ctg	2655
	Ile Ile Arg Thr Ile Gly Pro Val Trp Asp Gly Asn Glu Val Trp Leu	
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	Thr Met Phe Ser Gly Met Tyr Leu Pro Leu Phe Leu Val Leu Val Ser	
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	Leu Ile Met Arg Val Val Gly Leu Glu Trp Arg Lys Lys Val Asp Asp	
30	95 100 105	
	cct cgt tgg caa aag tgg tct gac cgg gcc atc ttt att ggt tct tgg	2847
	Pro Arg Trp Gln Lys Trp Ser Asp Arg Ala Ile Phe Ile Gly Ser Trp	
35	110 115 120	
	act cca ccg ctg atg tgg gga ttc atc ttc gcc aat att ttc aag ctt	2895
	Thr Pro Pro Leu Met Trp Gly Phe Ile Phe Ala Asn Ile Phe Lys Leu	
40	125 130 135 140	
	gca tgc cca tca agg cgg atc aca cca tcg atg ctg cag tgg ctc tgc	2943
	Ala Cys Pro Ser Arg Arg Ile Thr Pro Ser Met Leu Gln Trp Leu Cys	
45	145 150 155	
	tgt gca atg ttc aac gtc ttc gcc atc ctg ggt gca ctt gca ttc act	2991
50		
55		

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Cys Ala Met Phe Asn Val Phe Ala Ile Leu Gly Ala Leu Ala Phe Thr

160

165

170

5

gcg ctg ttc gct ctt cat ggc ctt gca ttc atc cgc ctg aaa act gct 3039

Ala Leu Phe Ala Leu His Gly Leu Ala Phe Ile Arg Leu Lys Thr Ala

175

180

185

10

ggc cgg gtg cgc acc gat ggc ggc aag gca gct cca gta gtc gca ctt 3087

Gly Arg Val Arg Thr Asp Ala Ala Lys Ala Ala Pro Val Val Ala Leu

190

195

200

15

ctt gct ggc gtg act ggt gga cct ttc gtg ttg tgg gct gcc atc gca 3135

Leu Ala Ala Val Thr Gly Gly Pro Phe Val Leu Trp Ala Ala Ile Ala

205

210

215

220

20

tac ggc cgt tcc tgg tcc tgg atc ctc gca gtg ctg atc atc gca ggc 3183

Tyr Gly Arg Ser Trp Ser Trp Ile Leu Ala Val Leu Ile Ile Ala Ala

225

230

235

25

gtt ctc ggt gga gct ttc gca ctg atc aaa gac cgc gat gga tta agc 3231

Val Leu Gly Gly Ala Phe Ala Leu Ile Lys Asp Arg Asp Gly Leu Ser

240

245

250

30

ttc ctg tcc act tcc gtc gct gtc atc ggt gta gtt gca ctg ctg ttt 3279

Phe Leu Ser Thr Ser Val Ala Val Ile Gly Val Val Ala Leu Leu Phe

255

260

265

35

agt tcc cta ttc ccc aac gtc atg cca aca acg ctt gcc gat ggc gtg 3327

Ser Ser Leu Phe Pro Asn Val Met Pro Thr Thr Leu Ala Asp Gly Val

270

275

280

40

act gga tat ttg gaa cgc ctc cgc aag cca cta cgc att gac cat cct 3375

Thr Gly Tyr Leu Glu Arg Leu Arg Lys Pro Leu Arg Ile Asp His Pro

285

290

295

300

45

gac ttg gac cgc cac tgt gat cgc acc gct ggt tgt cct cta cca agg 3423

Asp Leu Asp Arg His Cys Asp Arg Thr Ala Gly Cys Pro Leu Pro Arg

50

55

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	305	310	315	
5	ctg gac cta ctg ggt gtt ccg caa acg act tca cgc cga gcc agt gtc			3471
	Leu Asp Leu Leu Gly Val Pro Gln Thr Thr Ser Arg Arg Ala Ser Val			
	320	325	330	
10	tgc cta aaa gtt gga aaa att gag tac taaatctgac gctcggcta			3518
	Cys Leu Lys Val Gly Lys Ile Glu Tyr			
	335	340		
15	gtcgccgcac agggcccgtc gatccgcggc ttttgccct atccctgct acccgccgtt			3578
	gggtgataat cgcaggtgtt ctccaccgct tgaaaactct cgcgacagtc gcaatgggct			3638
20	tgtcatcgg ccagatggca gcgggcatca ttgaggttc gggaagtct ttgccccgaa			3698
	tggaactcat cgcgctcgcc atcacggtgg ttgtgcgcg acttcttgcg tgggcacagg			3758
25	atcggttcgg agcgcgcac gtcccagggt actgtggatc ttcgggagaa aaccctgcgg			3818
	cacctggcac aaagcgatcc ccgcaccatc gatcaagcct tgtggcgcac ccgtttgacc			3878
30	tctggccttg atggtttggg gccttacctc accggatttt tgccgcactg gccgccac			3936
35				
	<210> 4			
	<211> 341			
	<212> PRT			
40	<213> Brevibacterium lactofermentum			
	<400> 4			
45	Met Asp Leu Asn Thr Phe Trp Phe Ile Leu Ile Ala Phe Leu Phe Ala			
	1 5 10 15			
	Gly Tyr Phe Leu Leu Glu Gly Phe Asp Phe Gly Val Gly Ile Leu Ala			
50	20 25 30			
55				

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Pro Ile Ile Gly Lys Asp Ser Ala Ala Lys Asn Thr Ile Ile Arg Thr
35 40 45

Ile Gly Pro Val Trp Asp Gly Asn Glu Val Trp Leu Ile Val Ala Gly
50 55 60

Gly Ala Leu Phe Ala Ala Phe Pro Glu Trp Tyr Ala Thr Met Phe Ser
65 70 75 80

Gly Met Tyr Leu Pro Leu Phe Leu Val Leu Val Ser Leu Ile Met Arg
85 90 95

Val Val Gly Leu Glu Trp Arg Lys Lys Val Asp Asp Pro Arg Trp Gln
100 105 110

Lys Trp Ser Asp Arg Ala Ile Phe Ile Gly Ser Trp Thr Pro Pro Leu
115 120 125

Met Trp Gly Phe Ile Phe Ala Asn Ile Phe Lys Leu Ala Cys Pro Ser
130 135 140

Arg Arg Ile Thr Pro Ser Met Leu Gln Trp Leu Cys Cys Ala Met Phe
145 150 155 160

Asn Val Phe Ala Ile Leu Gly Ala Leu Ala Phe Thr Ala Leu Phe Ala
165 170 175

Leu His Gly Leu Ala Phe Ile Arg Leu Lys Thr Ala Gly Arg Val Arg
180 185 190

Thr Asp Ala Ala Lys Ala Ala Pro Val Val Ala Leu Leu Ala Ala Val
195 200 205

Thr Gly Gly Pro Phe Val Leu Trp Ala Ala Ile Ala Tyr Gly Arg Ser
210 215 220

Trp Ser Trp Ile Leu Ala Val Leu Ile Ile Ala Ala Val Leu Gly Gly

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	225	230	235	240
5	Ala Phe Ala Leu Ile Lys Asp Arg Asp Gly Leu Ser Phe Leu Ser Thr			
	245		250	255
10	Ser Val Ala Val Ile Gly Val Val Ala Leu Leu Phe Ser Ser Leu Phe			
	260		265	270
15	Pro Asn Val Met Pro Thr Thr Leu Ala Asp Gly Val Thr Gly Tyr Leu			
	275		280	285
20	Glu Arg Leu Arg Lys Pro Leu Arg Ile Asp His Pro Asp Leu Asp Arg			
	290		295	300
25	His Cys Asp Arg Thr Ala Gly Cys Pro Leu Pro Arg Leu Asp Leu Leu			
	305		310	315
30	Gly Val Pro Gln Thr Thr Ser Arg Arg Ala Ser Val Cys Leu Lys Val			
	325		330	335
35	Gly Lys Ile Glu Tyr			
	340			
40	<210> 5			
	<211> 19			
	<212> PRT			
	<213> Brevibacterium lactofermentum			
45	<220>			
	<221> UNSURE			
	<222> (18)			
50	<400> 5			
	Met Asp Val Val Asp Ile Ala Arg Trp Gln Phe Gly Ile Thr Ala Val			
	1	5	10	15
55				

Tyr Xaa Phe

5

<210> 6

<211> 20

10

<212> PRT

<213> Brevibacterium lactofermentum

15

<220>

<221> UNSURE

<222> (16,17)

20

<400> 6

Met Asp Leu Asn Thr Phe Trp Phe Ile Leu Ile Ala Phe Leu Phe Xaa

1

5

10

15

25

Xaa Tyr Phe Leu

20

30

<210> 7

<211> 20

<212> DNA

35

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer for PCR

40

<220>

<221> misc_feature

45

<222> (9,12)

<223> n=a or c or g or t

<400> 7

50

atggaygtng tngayatygc

20

55

<210> 8

<211> 20

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer for PCR

<400> 8

caraargtrt tvarrtccat

20

<210> 9

<211> 24

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer for PCR

<400> 9

cgccagggtt ttcccagtcg cgac

24

<210> 10

<211> 24

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer for PCR

<400> 10

gagcggataa caatttcaca cagg

24

Claims

1. A DNA fragment encoding a polypeptide defined in the following (A) or (B);

(A) a polypeptide which has an amino acid sequence shown in SEQ ID NO: 2 of Sequence Listing,

(B) a polypeptide which has an amino acid sequence shown in SEQ ID NO: 2 of Sequence Listing comprising

substitution, deletion, insertion, addition or inversion of one or a plurality of amino acid residues in the amino acid sequence, and can constitute a protein exhibiting cytochrome bd type quinol oxidase activity together with a subunit II of cytochrome bd type quinol oxidase having an amino acid sequence shown in SEQ ID NO: 4.

- 5 2. A DNA fragment encoding a polypeptide defined in the following (C) or (D);

(C) a polypeptide which has an amino acid sequence shown in SEQ ID NO: 4 of Sequence Listing,

(D) a polypeptide which has an amino acid sequence shown in SEQ ID NO: 4 of Sequence Listing comprising substitution, deletion, insertion, addition or inversion of one or a plurality of amino acid residues in the amino acid sequence, and can constitute a protein exhibiting cytochrome bd type quinol oxidase activity together with a subunit I of cytochrome bd type quinol oxidase having an amino acid sequence shown in SEQ ID NO: 2.

3. A DNA fragment encoding a polypeptide defined in the following (A) or (B), and a polypeptide defined in the following (C) or (D);

(A) a polypeptide which has an amino acid sequence shown in SEQ ID NO: 2 of Sequence Listing,

(B) a polypeptide which has an amino acid sequence shown in SEQ ID NO: 2 of Sequence Listing comprising substitution, deletion, insertion, addition or inversion of one or a plurality of amino acid residues in the amino acid sequence, and can constitute a protein exhibiting cytochrome bd type quinol oxidase activity together with a subunit II of cytochrome bd type quinol oxidase having an amino acid sequence shown in SEQ ID NO: 4,

(C) a polypeptide which has an amino acid sequence shown in SEQ ID NO: 4 of Sequence Listing,

(D) a polypeptide which has an amino acid sequence shown in SEQ ID NO: 4 of Sequence Listing comprising substitution, deletion, insertion, addition or inversion of one or a plurality of amino acid residues in the amino acid sequence, and can constitute a protein exhibiting cytochrome bd type quinol oxidase activity together with a subunit I of cytochrome bd type quinol oxidase having an amino acid sequence shown in SEQ ID NO: 2.

4. The DNA according to claim 1, which is a DNA defined in the following (a) or (b):

(a) a DNA having a nucleotide sequence corresponding to nucleotide numbers 933 to 2483 in the nucleotide sequence depicted in SEQ ID NO: 1 in Sequence Listing; or

(b) a DNA which is hybridizable with the nucleotide sequence of above (a) under a stringent condition, and which codes for a polypeptide which can constitute a protein exhibiting cytochrome bd type quinol oxidase activity together with a subunit II of cytochrome bd type quinol oxidase having an amino acid sequence shown in SEQ ID NO: 4.

5. The DNA according to claim 2, which is a DNA defined in the following (c) or (d):

(c) a DNA having a nucleotide sequence corresponding to nucleotide numbers 2476 to 3498 in the nucleotide sequence depicted in SEQ ID NO: 3 in Sequence Listing; or

(d) a DNA which is hybridizable with the nucleotide sequence of above (c) under a stringent condition, and which codes for a polypeptide which can constitute a protein exhibiting cytochrome bd type quinol oxidase activity together with a subunit I of cytochrome bd type quinol oxidase having an amino acid sequence shown in SEQ ID NO: 2.

6. The DNA according to claim 3, which comprising a DNA defined in the following (a) or (b), and a DNA defined in the following (c) or (d):

(a) a DNA having a nucleotide sequence corresponding to nucleotide numbers 933 to 2483 in the nucleotide sequence depicted in SEQ ID NO: 1 in Sequence Listing; or

(b) a DNA which is hybridizable with the nucleotide sequence of above (a) under a stringent condition, and which codes for a polypeptide which can constitute a protein exhibiting cytochrome bd type quinol oxidase activity together with a subunit II of cytochrome bd type quinol oxidase having an amino acid sequence shown in SEQ ID NO: 4; and

(c) a DNA having a nucleotide sequence corresponding to nucleotide numbers 2476 to 3498 in the nucleotide sequence depicted in SEQ ID NO: 3 in Sequence Listing; or

(d) a DNA which is hybridizable with the nucleotide sequence of above (c) under a stringent condition, and which codes for a polypeptide which can constitute a protein exhibiting cytochrome bd type quinol oxidase activity together with a subunit I of cytochrome bd type quinol oxidase having an amino acid sequence shown

in SEQ ID NO: 2.

7. The DNA fragment of claim 1, which has a nucleotide sequence comprising nucleotides of the nucleotide numbers 933 to 2483 in the nucleotide sequence shown in SEQ ID NO: 1.
- 5 8. The DNA fragment of claim 2, which has a nucleotide sequence comprising nucleotides of the nucleotide numbers 2476 to 3498 in the nucleotide sequence shown in SEQ ID NO: 1.
9. The DNA fragment of claim 3, which has a nucleotide sequence comprising nucleotides of the nucleotide numbers 933 to 3498 in the nucleotide sequence shown in SEQ ID NO: 1.
- 10 10. An expression vector comprising a DNA molecule according to any one of claims 1 to 9.
11. A procaryotic host cell comprising one or more expression vector(s) according to claim 10.
- 15 12. A method for the production of a protein having cytochrome bd type quinol oxidase activity comprising culturing the procaryotic host cell according to claim 11 and isolating said protein from said host or culture.

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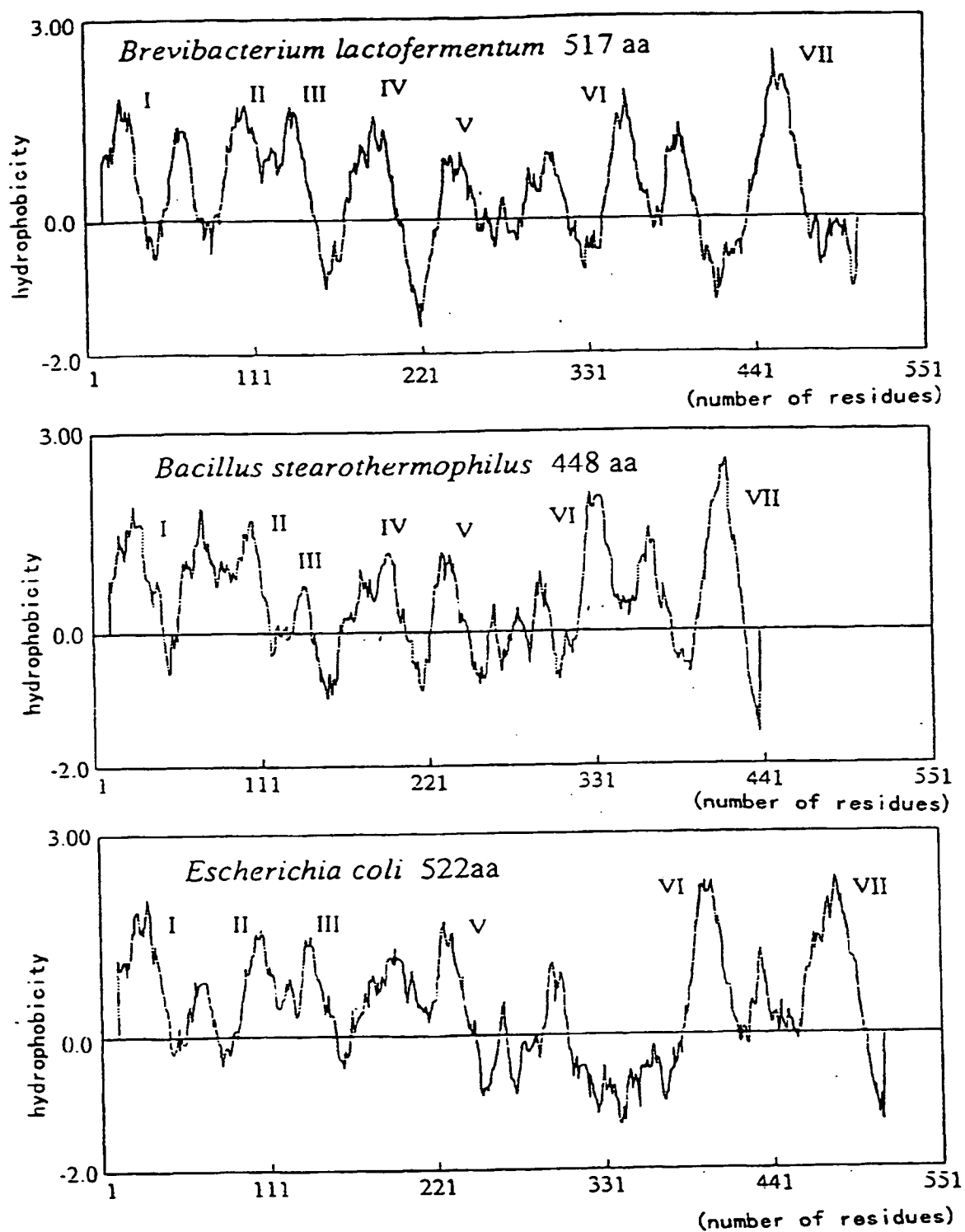


Fig. 1

[illegible]

Fig. 2

Br.l cydB 1:M-----DLNTFWFLIAFLFAGYFLLEGDFGVGILAPIIGKDSAAKNTIIRTIGPV 52
 B.st cydB 1:MTLEVIGISVLWLEFGYIIVASIDFGAGFFSV-YSHWANQQHILHR-IIQRYLSPV 55
 E.co cydB 1:MIDYEVL-RFIWMLLVGVLLIGFAVTDGFDMGVGMLTRFLGRNDTERRIMINSIAPH 56
 I

Br.l cydB 53:WDGNEVWLIVAGGALFAAFPEWYATMFSGMYLPLFLVLVSLIMRVVGLEWRKKVDDPRWQ 112
 B.st cydB 56:WEVTNVFLVFFVVGIVGFFPKTAYYYGSILLVPASIAIVLLAIRGSYYAFH-TYGETER- 113
 E.co cydB 57:WDGNQVWLITAGGALFAAWPMVYAAAFSGFYVAMILVLASLFFRPVGFDIRSKIEETRWR 116
 II

Br.l cydB 113:KWSORAIFIGSWTPPLMWGFIFANIFKLACPSRRITPSMLQWLCCAMFNVFALGALAFTA173
 B.st cydB 114:-NWYLLAYGLTGLFIPASLSIVLTISE-GGFVERNAAGVALDYGKLFASPLSWSVLLSVT172
 E.co cydB 117:NMWDWGIFIGSFVPPLVIGVAFGNLLQ-GVPFNVDEYLRLLYYTGNFFQLNPFGLLAGVVS176
 III IV

Br.l cydB 174:LFALHGLAFIRLKTAGRVRTDAAKAAPVVALLAAVTGGPFVLWAAIAYGRSW----- 225
 B.st cydB 173:SVLYISAVFLTYADAAGDEQARALLRRYALLWSGPTMLSALLIIYQLRYHN----- 224
 E.co cydB 177:VGMIITQGATYLQMRVTGELHLRTRATAQVAALVTLVCFALAGVWVMYGDYGVVKSTMD 236
 V

Br.l cydB 226:-----SWILAVLIIAAVLGGAFALIKDRDGLSFLS 255
 B.st cydB 225:-----PEHYDNLWNVAWMLVISFLFFVITVWLLGRQRRFGW 260
 E.co cydB 237:HYAASNPLNKEVVREAGAWLVNFNNTPIWAIPALGVVLPLLTILTARMDKAAWAFV 294
 VI

Br.l cydB 256:TSVAVIGVVALLFSSLPNVMPPTLADGVTGYLERLRKPLRIDHPDLDRHCORTAGCPLP 315
 B.st cydB 261:AFIALLFQYAFAYAYGISHYPYLLPY--LTIYDGFTNETMAMALIVAFIAGLLLLIP- 317
 E.co cydB 295:SSLTLACIILTAGIAMFPFVMPSSSTMNASLTMWDATSSQTLNVMTWVAVVLVPIILLY 354
 VII VIII

Br.l cydB 316:RLDLLGVPQTTSRRASVCLKVGKIEY 341
 B.st cydB 318:SLYLLMRLFLFNKAYVKGKWEKKG 342
 E.co cydB 355:TAWCYWKMFGKITKEDIERNTHSLY 379

Fig. 3